Author's response to reviews

Title: Increased expression of oxyproteins in the optic nerve head of an in vivo model of optic nerve ischemia

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Author's response to reviews: see over
Dear Emilie Aime

Executive Editor

BMC Ophthalmology

We are enclosing manuscript entitled, "Increased expression of oxyproteins in the optic nerve head of an in vivo model of optic nerve ischemia" for consideration of publication in BMC Ophthalmology. The manuscript has been revised according the reviewers’ suggestions. We hope the revised manuscript will meet the requirements for publication of the 'BMC Ophthalmology'. We believe that this work will be of interest to your readers. Thank you for your kind considerations and I am looking forward to your comment.

Sincerely yours,

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Kim and colleagues reported increases expression of oxyprotein induces retinal ganglion cell and inner nuclear cell loss. The experimental design appears appropriate and data is interesting. However, the results need major revision. It would be very useful if the authors would have more clarification in some of the methodology and interpretation of the results(see below). The significant of the work is limited without clarification.

Introduction
Please expand the introduction and summarize the characteristic function of ET-1 and relations with oxidative stress.

We added contents about the characteristic function of ET-1 and relations with oxidative stress as below. (page 3, line 21 ~ page 4 line 6)

Increased levels of ET-1 have been known to lead to a reduction in blood flow in both the choroid and the optic nerve head.[19] ET-1 constricts vessels both directly and indirectly by increasing the sensitivity to other vasoconstrictive hormones such as norepinephrine, 5-hydroxytryptamine, and angiotensin-II. An increase in circulating ET-1 markedly reduces blood flow in the eye.[20] If the concentration of ET-1 is even higher, it causes vasospasm.[21] The stimulation of ET receptors on smooth muscle cells or pericytes increases cytoplasmic calcium, both by influx into the cell, as well as by liberation of calcium from the internal storage.[22] High levels of ET-1 in the eye cause pro-inflammatory cytokine overproduction and oxidative stress pathway activation, as well as reduced trophic support and oxygen delivery to the retina.[23]

Material and methods
What methods for TUNEL staining?

We performed TUNEL staining as below and added the method for TUNEL staining in the manuscript. (page 5, line 23 ~ 27)

TUNEL staining was performed using an ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore, USA). The sections were treated with proteinase K (DAKO, USA) and blocked by hydrogen peroxide. Following the washes, the sections were incubated with an enzyme buffer containing terminal deoxynucleotidyl transferase and dUTP. The apoptotic cells were detected by DAB substrate chromogen system (DAKO).
Results
The authors concluded that cell numbers in retinal ganglion cell layer, INL and ONL decreased. Does it base on cell counts or thickness of the layer?

Our results based on thickness of the layer. With destruction of intercellular structure, intercellular space has increased. Viewed in this light if the layer thickness decrease and intercellular space increase, we can consider that decreased cell numbers.

We corrected the manuscript as below. (page 6, line 6 ~ 7)

- The intercellular structures in each retinal cell layer were destroyed and intercellular space loosened, the cell number of cell layer seems to have decreased.

Arrows on Fig 3II, III and IV for apoptotic positive cell is not convincing.

II, III, IV have few cell as compared with I. So we tried to show that the TUNEL stain nearly unstained in II, III, IV as compared with I.

Discussion:
No expression of ET-1 was shown in the retina, yet cell loss was concluded in retinal layers. Please give more explanation.

It probably means that oxyprotein.

We collected and analysed optic nerve head tissue in which protrudes into the globe for optic nerve head oxyblot. It might mainly consist of the surface nerve fiber layer and the prelaminar layer. The layer includes mainly axon and some deep retinal layer. That structure is difference form surface retinal layer. So if oxyprotein more expressed in the axon, the oxyblot result is difference from the thin axon of existing retinal layer. And because oxyblot is quantitative method, a certain amount is needed for detection. Therefore, we can’t make a conclusion that no expression is no existence.

We added the contents as below. (page 8, line 9 ~ 18)

- In our results, there was no expression of oxyproteins in the retina by oxyblot, yet cell loss was observed in retinal layers. In this study, we analyzed separation of the optic nerve head from the retinal layer. For this analysis, we collected optic nerve head tissue, which protrudes into the globe. The optic nerve head consists mainly of the surface nerve fiber layer and the prelaminar layer. Those layers include mainly axons and some deep retinal layers. The structure of the retina containing whole layer is different from that of the surface retinal layers. Therefore, if oxyprotein is expressed more in the axons, the oxyblot result will be different from that of the thin axons of the existing
Since oxyblot is a quantitative method, a certain amount of oxyprotein is needed for detection. Therefore, the absence of detection can be attributed to a low amount of oxyprotein in the tissue. To date, there have been no studies regarding whether oxyprotein can migrate along the axon.

Seemingly thickness of the INL and ONL are greatly reduced compared to control, yet apoptotic cell deaths were not revealed. Any explanation of mechanism of the cell loss?

*We think that various mechanism involved in the cell loss*

Ischemia-induced oxidative stress at the retrobulbar area can damage the retina and optic nerve. We injected endothelin from the outside but the damage of inner retina was more severe. The result suggested that the more important mechanism is the indirect vascular effect rather than the direct toxic effect. The excessive reactive oxygen species induced by oxidative damage directly trigger many insults in the retina, including mitochondrial dysfunction, activation of the apoptosis pathway, induction of neurotoxins, weakening of the neuroprotective functions of glial cells, and activation of immune-mediated neuronal injury. The retinal ganglion cells are damaged through these various pathways.

**Level of interest:** An article whose findings are important to those with closely related research interests  
**Quality of written English:** Acceptable  
**Statistical review:** No, the manuscript does not need to be seen by a statistician.  
**Declaration of competing interests:** I declare that I have no competing interest

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**Reviewer's report**  
**Title:** Increased expression of oxyproteins in the optic nerve head of an in vivo model of optic nerve ischemia  
**Version:** 1 **Date:** 21 September 2012  
**Reviewer:** A. Wegner  
**Reviewer's report:**  
There are no legends to the figures and table.

The legends are as follows.

**Figure 1.** Results of OxyBlot protein detection (A) and relative densitometric values of oxyprotein expression in the optic nerve head (B, C). Blotting was
semi-quantified using densitometry. To compare the results of OxyBlot protein oxidation detection, relative densitometric values of treated groups were compared to control groups. Endothelin-1 (0.1 μg/day) was delivered to the perineural region of the anterior optic nerve by osmotically-driven minipumps for two (II), four (III), and eight weeks (IV). As a control, balanced salt solution was delivered for two (Ia) and eight weeks (Ib). There was a significant increase in the expression of oxyprotein after two weeks of endothelin-1 administration (p<0.001, Mann Whitney U test). However, there was no difference between the two groups at eight weeks.

Figure 2. Retina histological findings (H&E staining, x 200). An endothelin-1 dosage of 0.1 μg/day was delivered for two (II), four (III), and eight weeks (IV), and balanced salt solution was delivered for eight weeks (Ib) to the perineural region of the anterior optic nerve. Compared to Group Ib, the number of cells in the retinal ganglion cell layer (long arrow), the inner nuclear layers (short arrow), and the outer nuclear layer (green arrowhead) were remarkably decreased in Groups II, III, and IV. Vacuoles without a nucleus (empty arrowhead) were found in endothelin-1-treated groups. In addition, thinning of the inner and outer nuclear layers and the inner and outer plexiform layers was noted. The outer limiting membrane, composed of the zonula adherens, exhibited loosening and widening (yellow arrowhead).

Figure 3. Retina histological findings (TUNEL staining, x 200). An endothelin-1 dosage of 0.1 μg/day was delivered for two (II), four (III), and eight weeks (IV) and balanced salt solution was delivered for eight weeks (Ib) to the perineural region of the anterior optic nerve. Apoptotic cells (gray) were observed mainly
in the ganglion cell layer (long arrow) and inner nuclear layer (short arrow) in Group Ib. However, very few apoptotic cells were observed in Groups II, III, and IV.

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I declare that I have no competing interests